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Genetic Instability in Epithelial Tissues at Risk for Cancer

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ABSTRACT: Epithelial tumors develop through a multistep process driven by genomic instability frequently associated with etiologic agents such as prolonged tobacco smoke exposure or human papilloma virus (HPV) infection. The purpose of the studies reported here was to examine the nature of genomic instability in epithelial tissues at cancer risk in order to identify tissue genetic biomarkers that might be used to assess an individual's cancer risk and response to chemopreventive intervention. As part of several chemoprevention trials, biopsies were obtained from risk tissues (i.e., bronchial biopsies from chronic smokers, oral or laryngeal biopsies from individuals with premalignancy) and examined for chromosome instability using *in situ* hybridization. Nearly all biopsy specimens show evidence for chromosome instability throughout the exposed tissue. Increased chromosome instability was observed with histologic progression in the normal to tumor transition of head and neck squamous cell carcinomas. Chromosome instability was also seen in premalignant head and neck lesions, and high levels were associated with subsequent tumor development. In bronchial biopsies of current smokers, the level of ongoing chromosome instability correlated with smoking intensity (e.g., packs/day), whereas the chromosome index (average number of chromosome copies per cell) correlated with cumulative tobacco exposure (i.e., pack-years). Spatial chromosome analyses of the epithelium demonstrated multifocal clonal outgrowths. In former smokers, random chromosome instability was reduced; however, clonal populations appeared to persist for many years, perhaps accounting for continued lung cancer risk following smoking cessation.

KEYWORDS: chromosome instability; epithelial cells; aerodigestive tract; chemoprevention; cancer risk

THE NEED FOR BIOMARKERS OF CANCER RISK AND RESPONSE TO INTERVENTION

Epithelial cancers remain a major health challenge in the world. Despite improvements in staging and the application and integration of surgery, radiotherapy, and chemotherapy, the 5-year survival rate for individuals with lung cancer is only about 15%.[1] Even if strategies for early detection are successful and lung cancers are detected at a stage where local tumor resection and treatment is curative, these patients will still be at significant risk for developing second primary tumors

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associated with the problem of field cancerization.[2] Similarly, for individuals with a first head and neck primary tumor, even if the first malignancy is successfully treated, the risk of developing a second primary in the tobacco smoke-exposed field is approximately 40%.[3] Similar cancer risk estimates exist for individuals who exhibit severe dysplasia in premalignant epithelial lesions.[4] For these reasons, it is important to focus on chemopreventive strategies to prevent the development of epithelial malignancies.

Several problems confront chemoprevention trials designed to identify efficacious agents.[5] First, chemoprevention trials with cancer incidence as a primary endpoint require tens of thousands of subjects and tens of years of intervention and follow-up for statistical evaluation. For example, a recently reported trial involved 30,000 subjects and required 10 years in order to examine the impact of prevention strategies on lung cancer development, only to find a possible increased lung cancer incidence in current smokers who received β-carotene.[6]

The problem of large, long-term trials results from the difficulty in identifying individuals at highest cancer risk who might best benefit from chemopreventive intervention. For example, 20 pack-year smokers, while known to be at relatively increased risk for developing lung cancer, have approximately a 10% lifetime risk for developing lung cancer.[7] This seriously limits the number of potentially useful strategies that can be clinically explored. A second problem facing chemoprevention trials is that little is known about what agents are likely to have efficacy, and even less is known regarding proper doses, schedules, and durations of treatment. Part of the reason for this problem is that too little is known about the physiologic processes that drive epithelial cancer development.

In order to reduce the number of subjects and the time required to carry out chemoprevention trials and thus allow the exploration of multiple prevention strategies, two types of advances are necessary. First, it is important to identify individuals at significantly increased cancer risk who might best benefit from different types of intervention. Second, in order to allow the rapid identification of agents, doses, and schedules of potentially efficacious agents, it is necessary to identify and validate surrogate endpoints of response that indicate whether the agents are having a positive impact on the target tissue during the chemopreventive intervention.

One approach to identifying individuals at increased aerodigestive tract cancer risk is to explore epidemiologic features of potential subjects. Molecular epidemiologic studies are beginning to identify intrinsic host factors that place some individuals at increased cancer risk, especially those with a chronic smoking history.[8] Most intrinsic factors identified thus far reflect levels of carcinogen metabolism, repair capabilities of the host following DNA damage, and other measures of intrinsic cellular sensitivity to mutagens. While these factors can provide statistically significant risk ratios in case-control studies that are controlled for tobacco exposure, the detected risk ratios usually fall in the range of 1.5 to 10. Unfortunately, this is not sufficient for the individualization of treatment and is not sufficiently high to significantly reduce the numbers of subjects required for chemoprevention trials with cancer incidence as the primary endpoint.

Another approach to identifying individuals at increased cancer risk is to directly examine the target tissue of individuals with known carcinogen exposure (e.g., chronic tobacco smoke exposure), who have evidence of target organ dysfunction

(e.g., chronic obstructive pulmonary disease, changes in voice quality), or who have clinical evidence of premalignancy (e.g., bronchial metaplasia/dysplasia, oral leukoplakia/erythroplakia, cervical intraepithelial neoplasia). The conventional standard for assessing cancer risk in these situations is the degree of histological change. However, while individuals who show moderate to severe dysplasia are known to be at increased cancer risk when compared to individuals with lesser histologic changes, it is often difficult to distinguish reactive changes to carcinogenic insult from initiated and progressing lesions. Similarly, upon cessation of carcinogenic insult, histologic changes may reverse yet cancer risk may continue for many years. For example, while smoking cessation is associated with decreased bronchial metaplasia;[9] increased lung cancer risk continues for many years beyond smoking cessation.[10] In fact, nearly half the newly diagnosed lung cancer cases in the USA occur in former smokers.[11]

The development of assays to identify individuals at high epithelial cancer risk and to directly assess response to intervention in the target tissue is therefore an important research goal. Such assays should be objective and easily quantifiable and, if possible, minimally invasive. Moreover, they should reflect both the disease process and the targeted pathway and thereby be useful in assessing risk and monitoring response to intervention as well as directly testing the hypothesized mechanism of action of the chemopreventive strategy.

In the chemoprevention setting it is important to recognize that one does not know the location of the future cancer. Thus, assays must necessarily be carried out on random biopsies of the field at risk. Even if there are clinically evident premalignant lesions, this does not mean that this is the likely site for a future malignancy. For example, nearly half of the cancers that develop in individuals with oral leukoplakia arise away from the original index lesion. Similarly, since many newly diagnosed lung cancers arise in the peripheral parts of the lung (e.g., adenocarcinomas), especially in former smokers, and since endobronchoscopy predominantly accesses central components of the lung, it is important to identify biomarkers that can reflect global processes ongoing in the target epithelial field associated with increased cancer risk. Their discovery requires a better understanding of the tumorigenesis process in epithelial fields at cancer risk.

THE RATIONALE FOR STUDYING GENOMIC INSTABILITY AS A MARKER OF RISK

Tumors of the aerodigestive tract have been proposed to reflect a "field cancerization" process whereby the whole tissue is exposed to carcinogenic insult (e.g., tobacco smoke) and is at increased risk for multistep tumor development.[12,13] Several types of clinical and laboratory data support this notion, including the frequent occurrence of synchronous primary and subsequent second primary tumors in the aerodigestive tract (frequently exhibiting dissimilar histologies as well as distinct genetic signatures[14–16]) and the presence of premalignant lesions that precede and/or accompany the tumor in the exposed tissue field.[17] The notion of a multistep tumorigenesis process is further supported by serial clinical and histologic evaluations of

target tissue or exfoliated cells where increasing degrees of histological abnormalities are observed over time.[18]

A working model for aerodigestive tract tumorigenesis is illustrated in FIGURE 1. Tumorigenesis in the face of carcinogenic exposure likely involves a chronic process of tissue injury and wound healing. DNA damage induced by the carcinogen is likely fixed into permanent genetic changes (e.g., chromosome damage, chromosome nondisjunction, gene mutation, gene deletion, etc.) during the process of proliferation. This damage would be expected to be distributed throughout the exposed tissue field leading to a background of generalized genomic damage (depicted in FIGURE 1 as a background mat of increasing density). Chronic injury and repair likely leads to the accumulation of cells with increasing amounts of genetic changes as well as the outgrowth of abnormal clones (triangles in FIGURE 1) carrying an accumulation of genetic changes important for selective survival, dysregulated growth, and preferential epithelial take-over by initiated clones (see FIGURE 2).

Cellular and molecular evidence for the field carcinogenesis and multistep tumorigenesis model comes from many laboratories.[19,20] With the advent of a wide array of molecular technologies, a large number of specific molecular genetic and epigenetic changes involving specific oncogenes, tumor suppressor genes, cell regulatory genes, and repair genes have now been described for aerodigestive tract cancers. The identification of these specific molecular changes have now provided probes to explore specific events occurring in premalignant lesions adjacent to aerodigestive tract tumors.[21–24] Frequently, these premalignant lesions showed a subset of the same molecular changes found in the associated tumor, suggesting that these lesions might represent precursor lesions for the associated tumors (i.e., a manifestation of

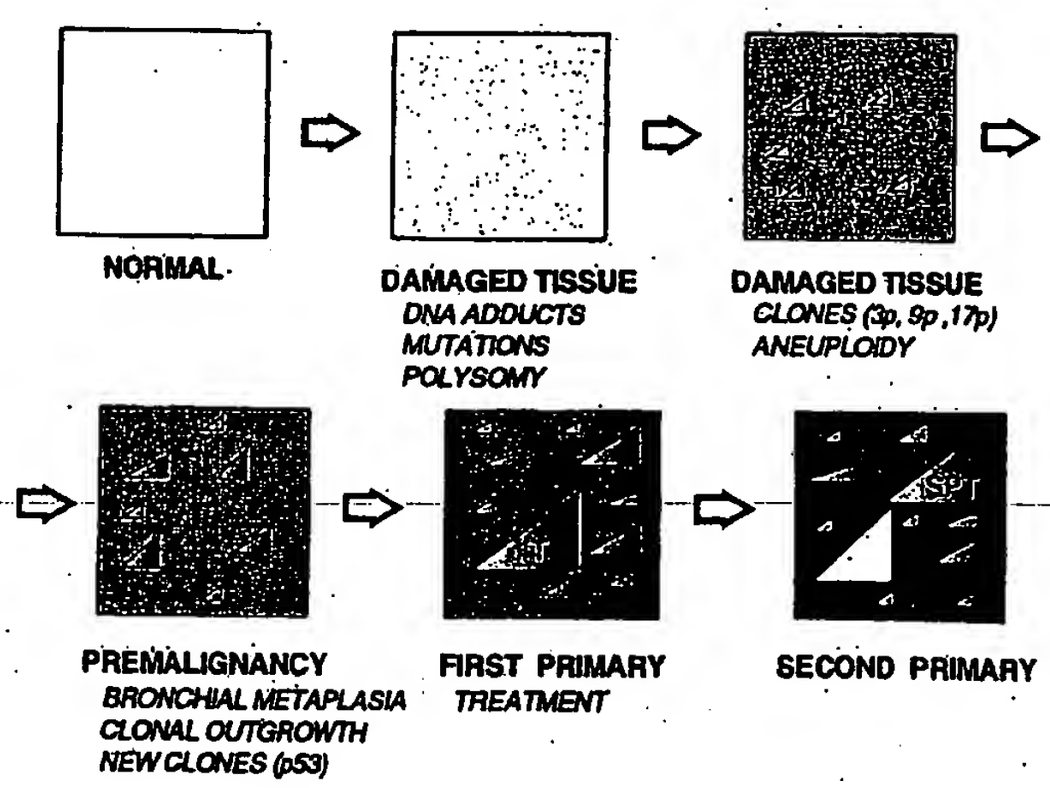

FIGURE 1. Field cancerization and multistep tumorigenesis.

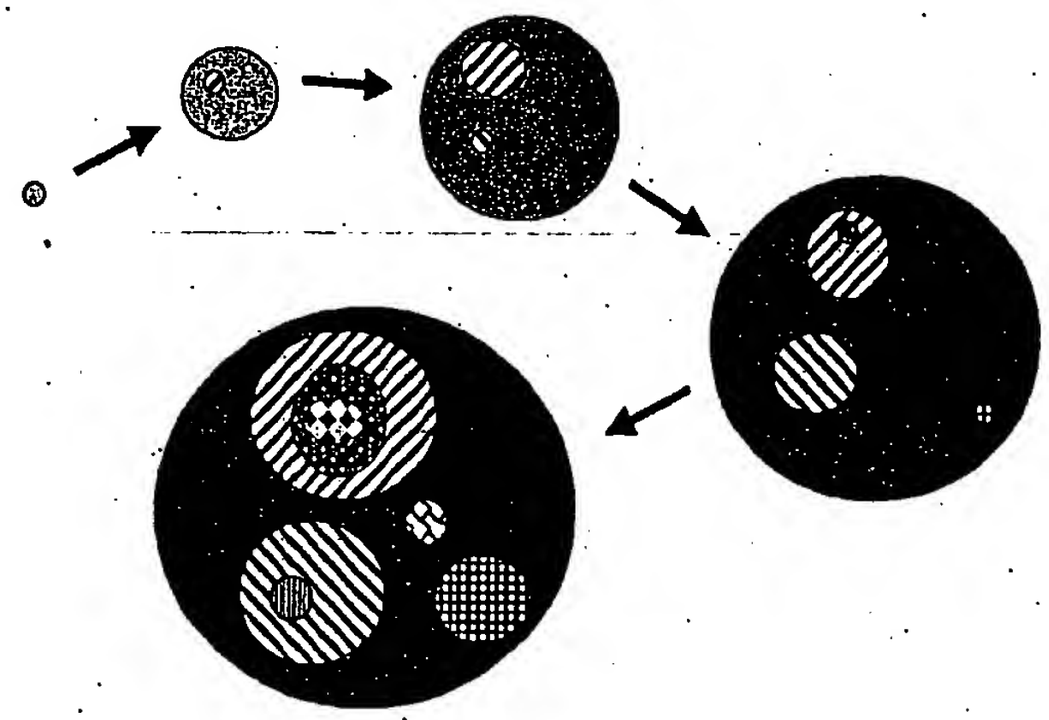

FIGURE 2. Multiple focal clonal evolution during multistep tumorigenesis.

a multistep tumorigenesis process). For example, studies of the premalignant lesions adjacent to head and neck tumors have provided evidence for a gradual accumulation of genetic alterations accompanied by evidence for dysregulation of cellular control mechanisms (e.g., alterations in expression of PCNA, EGFR, TGF-β, p53, and cyclin D1).[25–28]

These types of studies have now also been applied to the target epithelium of individuals at increased risk for aerodigestive tract cancer (i.e., individuals with a chronic smoking/alcohol history and/or prior aerodigestive tract cancer). Several groups (using polymerase chain reaction, PCR, analysis of microdissected epithelium) have now demonstrated the presence of clonal outgrowths in the target premalignant epithelium of individuals at increased risk for cancer.[29–31] For example, examination of bronchial biopsies derived from individuals with a 20 pack-year smoking history demonstrated that 76% of the cases showed evidence for LOH (3p14, 9p21, or 17p13) in at least one of six lung biopsy sites. On a per site basis, some form of LOH was observed in 25% of the sites examined.[29]

If aerodigestive tract cancer development reflects a field cancerization process involving multistep events, then risk and response information should be able to be derived from random biopsies or exfoliated cells from the field at risk or from assessments of tissue undergoing similar processes. Hypothetically, lesions exhibiting the greatest degree of genomic instability, clonal outgrowth, and abnormal epithelial regulation would be at the highest relative aerodigestive tract cancer risk. Similarly, an active chemopreventive intervention might be expected to decrease these manifestations of risk. Reduced risk manifestations include decreased levels of ongoing genetic instability, decreased frequency of clonal outgrowths, and increased epithelial growth regulation.

THE MEASUREMENT OF CHROMOSOME INSTABILITY USING CHROMOSOME *IN SITU* HYBRIDIZATION

Molecular genetic techniques, while extremely useful for detecting clonal changes in targets tissues, are somewhat limited in their ability to detect random genetic instability. Conventional cytogenetic assays are useful for detecting chromosome instability and clonal chromosome changes. However, they require numbers of dividing cells for karyotypic analysis that are difficult to attain in the setting of biopsies acquired during the course of a chemoprevention trial. A technique was therefore needed that would allow chromosome instability measurements in situations where few cells are available (e.g. small biopsies, brushings, or sputum samples) and where the target material might be fixed. It was also desirable to have a technique that would be adaptable to tissue sections, whereby spatial information could be retained and genotype/phenotype associations could be determined on the same or adjacent tissue sections. The technique of *in situ* hybridization (ISH) involves the use of DNA probes that recognize either chromosome-specific repetitive target sequences, chromosome single gene copy sequences, or sequences along the whole chromosome length or chromosome segments.[32] We have adapted the ISH technique for formalin-fixed, paraffin-embedded tissue sections and have applied it to a variety of tissues, including the aerodigestive tract.[33,34]

Using probes that label the centromere regions of specific chromosomes, this assay permits determination of the average chromosome number per cell for each specimen. This assay is also useful for detecting generalized chromosome instability during the tumorigenesis process. Normal diploid populations should have two copies of each autosomal chromosome and should rarely show three or more chromosome copies per cell (chromosome polysomy), especially in tissue sections where nuclear truncation results in an under-representation of chromosome copy number. Thus, the detection of cells with three or more chromosome copies would indicate the presence of chromosome instability.

To examine this technique's potential for characterizing the multistep tumorigenesis process in the aerodigestive tract, we measured the fraction of cells exhibiting three or more chromosome copies in apparently contiguous epithelial transitions from normal to hyperplastic to dysplastic to carcinomas, all on a single tissue slice of head and neck squamous cell carcinomas.[34] In these specimens, greater than 35% of the cases of adjacent "normal" epithelium, greater than 65% of the cases of hyperplastic epithelium, and greater than 95% of the dysplastic and tumor regions showed evidence of chromosome polysomy. Of interest, similar transitions of chromosome instability were observed with at least four different chromosome probes. Similar trends have also been observed in amenable tissue from other epithelial malignancies, including cervix, bladder, and breast.[35] These results thus suggested that the notions of field cancerization and multistep tumorigenesis might apply to several epithelial tissues and that measures of chromosome instability might be useful for monitoring this process.

In the situations described above, the premalignant lesions examined might be considered to represent epithelium at 100% risk of being in a cancer field, since they were located in the adjacent epithelium to the cancer. This then raises the question of the nature of genetic instability in the epithelium of individuals at increased risk

for developing cancer. To explore this issue, we obtained biopsies during the course of leukoplakia chemoprevention trials exploring the use of 13-*cis*-retinoic acid in reversing leukoplakia and probed them for genetic instability using *in situ* hybridization. In one retrospective study and in one prospective study of subjects with oral leukoplakia, the results indicate that those subjects whose pretreatment biopsies harbor relatively high levels of genomic instability (i.e., more than 3% of the cells examined showing at least 3 chromosome 9 copies per cell) have a significantly higher likelihood of suffering early onset of head and neck cancer.[36,37] Interestingly, half of the tumors that did develop occurred away from the biopsy site used to measure genetic instability. This result suggests that genomic instability measurements in carcinogen-exposed tissue can provide useful cancer risk estimates.

THE RELATIONSHIP BETWEEN TOBACCO EXPOSURE AND CHROMOSOME INSTABILITY

In recent years, the aerodigestive tract chemoprevention group at M.D. Anderson Cancer Center has initiated three sequential biomarker-associated chemoprevention trials involving chronic smokers with a greater than 20 pack-year smoking history. In each of these studies, endobronchial biopsies were obtained from six defined sites within the lung, including the carina and at bifurcation points at the upper, middle, and lower right lung and at the upper and lower left lung. Biopsies were obtained prior to and following chemopreventive intervention and were subjected to *in situ* hybridization analysis in addition to analyses for other biomarkers. The first important finding was that some degree of chromosome polysomy was evident in all lung sites examined, and this was observed independently of the particular chromosome probe utilized.[38] This finding supports the notion that random chromosome changes may be occurring throughout the exposed lung field.

In a second study, bronchial biopsies were obtained from individuals with a 20 pack-year smoking history. In this study, most of the subjects involved were current smokers.[39] Interestingly, all cases who showed metaplasia at one of six biopsy sites also showed chromosome polysomy in at least one biopsy site; overall, 88% of the sites showed some evidence of chromosome 9 polysomy.[40] Evidence for genetic instability was also detected in patients who did not show evidence of bronchial metaplasia in any of six biopsy sites despite a strong smoking history. In fact, more than 90% of the cases and more than 60% of the sites showed significant chromosome polysomy (i.e., at least three copies in at least 2 % of the cells examined). These results suggest that the lungs of long-term smokers show significant evidence of genetic instability, and this instability can be detected throughout the accessible bronchial tree, even when bronchial metaplasia is not evident.

These studies in current smokers has allowed us to examine the relationship between the levels of genetic instability detected and subject characteristics such as smoking status (current or former), smoking history, and lung tissue pathologic changes. Evaluable biopsy material has now been obtained from more than 108 current smokers, including more than 480 evaluable biopsy sites. The mean metaplasia index in these current smokers was 30.4%. For the total population studied, the median chromosome index for the bronchial biopsies was 1.41 (range, 1.04–1.61)

and the median chromosome polysomy index was 2.0% (range 0–8.7%). This can be compared to a mean chromosome index between 1.2–1.4 for lymphocytes and very rare chromosome polysomy. Interestingly, the intrasubject variability in chromosome instability was relatively low in most subjects and was less than the intersubject variability. These results suggested that chronic smokers harbor detectable chromosome instability throughout the accessible bronchial tree (supporting the field carcinogenesis notion) and that information from one biopsy site might yield representative information for the rest of the lung field.

Since most of the current smokers exhibited bronchial metaplasia in at least one of the biopsied sites, this allowed us to examine the relationship between chromosome instability and histologic changes, both on a site-by-site basis and on a per case basis. On a site-by-site basis, the chromosome indices of lesions showing squamous metaplasia were similar to those not showing metaplasia (i.e., median 1.43 vs. 1.43), and the degree of chromosome polysomy in metaplastic lesions were only slightly higher than in non-metaplastic sites (medians: 2.2% vs. 1.8%, respectively). Thus, the presence or absence of squamous metaplasia at a biopsy site does not necessarily correlate with the degree of underlying genomic instability. On the other hand, those subjects with metaplasia indices of at least 15% also showed higher levels of chromosome polysomy than did subjects with metaplasia index below 15% (medians: 2.4% vs. 1.8%, $p = 0.005$). Thus, these chromosome instability assessments in current smokers appeared to reflect a more global process in the lung field.

Tobacco exposure has been shown to significantly increase the risk of developing lung cancer, and the degree of risk is related to the extent of tobacco exposure. We were interested in determining the relationship between individuals' smoking history parameters and the levels of chromosome change found in their lungs following years of tobacco exposure. While there was significant intersubject variation for similar tobacco exposure histories, overall there was a significant correlation between the degree of chromosome polysomy and the intensity of ongoing tobacco exposure (packs/day, $p = 0.02$ on a per site basis) and with the extent of tobacco exposure (pack-years, $p = 0.003$). Thus the amount of chromosome polysomy reflects the intensity and extent of tobacco exposure. At the same time, individuals with similar smoking histories showed widely divergent amounts of chromosome polysomy, possibly reflecting differences in intrinsic sensitivity between subjects. There was also strong correlation between the chromosome index and the duration of the smoking history (smoking years) and total accumulated exposure (pack-years, $p = 0.0001$). These results suggest that tobacco exposure is associated with the initiation and accumulation of chromosome instability in the exposed lung; however individuals are differentially sensitive to carcinogenic insult. The working hypothesis is that those individuals who accumulate the highest degree of chromosome changes will be at the highest lung cancer risk.

Many of the bronchial biopsies from chronic smokers examined by *in situ* hybridization showed a rise in the chromosome index above that expected for a diploid cell population, especially in subjects with an extensive smoking history. The rise in chromosome index was also accompanied by an increase in the fraction of cells exhibiting at least 3 chromosome copies per cell. To determine if a rise in the tissue chromosome index was due to clonal expansion of populations with chromosome trisomy, the chromosome copy number and relative coordinates of each cell scored in

the bronchial epithelium was recorded and a spatial genetic map was created.[41] We then developed algorithms for calculating localized chromosome indices within the tissue. Since trisomic clones would have, on average, three chromosomes instead of two, those cells involved in neighborhoods with chromosome indices three-halves that of diploid populations could be marked as being part of a trisomic clone. Similarly, groups of cells with chromosome indices half that of diploid populations could be marked as being part of a monosomic clone. This allowed the generation of a second-order, two-dimensional genetic map representation of the bronchial epithelium showing the relative locations of cells involved in monosomic and trisomic clonal outgrowths. When adjacent tissue sections from the same bronchial biopsy were probed separately for different chromosomes, the detected clones appeared to occupy separate subregions of the epithelium. This result suggests that not only are the lungs of chronic smokers undergoing a process of genetic instability, they are experiencing the outgrowth of multiple clones throughout the exposed lung field, as postulated by the models shown in FIGURES 1 and 2. One advantage of this clonal approach is that the contribution of both monosomic and multisomic clones can be detected.

Since smoking cessation has been suggested to reduce the lung cancer risk, it was of interest to determine whether the levels of chromosome instability would decrease following smoking cessation. This question was possible to examine because our third sequential chemoprevention trial involved subjects who had discontinued smoking. So far, more than 220 subjects (more than 650 biopsies) who have quit smoking (mean 9.9 quit-years) have been evaluated for chromosome instability in their lungs. Despite the fact that the mean metaplasia index in this group is 5.8% (considerably less than that in current smokers), chromosome instability is still observed in the majority of subjects.[42] While the mean chromosome polysomy level is reduced to 1.0%, some individuals continue to show polysomy levels above 5%. Interestingly, while the overall chromosome polysomy levels were reduced in these individuals who stopped smoking, the mean chromosome index remained at about 1.4 with some individuals exhibiting chromosome indices as high as 1.8. Initial chromosome mapping studies suggest that while random chromosome instability seems to decrease following smoking cessation, the clonal outgrowths may remain for many years in the lung. The working hypothesis is that those individuals who show the greatest degree of remaining chromosome instability are at the highest lung cancer risk despite smoking cessation. Long-term follow-up on these subjects will be necessary to test this hypothesis.

SUMMARY AND CONCLUSIONS

Aerodigestive tract tumorigenesis appears to be a multistep process taking place throughout the tissue fields of exposure. When viewed in the context of chromosome changes, carcinogen exposure appears to be associated with the random acquisition of chromosome polysomy throughout the exposed field, the degree of which is related to the degree and extent of carcinogen exposure as well as to the instrinsic susceptibility of the exposed individual. Continued exposure leads to continued acquisition of new changes and, in association with chronic wound-healing processes, to the

accumulation of clonal outgrowths throughout the target tissue. Although the ultimate malignancy may occur in only one or few tissue sites, manifestations of the instability process that drives tumorigenesis is globally present in the tissue. Thus random biopsies may provide useful risk information for the exposed field as a whole. Even when carcinogen exposure is reduced or chemopreventive strategies are initiated and histologic manifestations of the tumorigenesis process subside, the genetic scars of prior exposure remain in the form of clonal outgrowths and may explain continued lung cancer risk in ex-smokers. Future chemoprevention strategies need to focus on reducing the degree of chromosome instability and on trying to eliminate residual abnormal clonal outgrowths in the aerodigestive tract. In this setting, the measurement of chromosome instability in the target tissue will be useful in assessing cancer risk as well as response to intervention.

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